

University of Groningen

## KCNJ10 mutations display differential sensitivity to heteromerisation with KCNJ16

Parrock, Sophie; Hussain, Sofia; Issler, Naomi; Differ, Ann-Marie; Lench, Nicholas; Guarino, Stefano; Oosterveld, Michiel J S; Keijzer-Veen, Mandy; Brilstra, Eva; van Wieringen, Hester

*Published in:*  
Nephron Physiology

*DOI:*  
[10.1159/000356353](https://doi.org/10.1159/000356353)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2013

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Parrock, S., Hussain, S., Issler, N., Differ, A.-M., Lench, N., Guarino, S., Oosterveld, M. J. S., Keijzer-Veen, M., Brilstra, E., van Wieringen, H., Konijnenberg, A. Y., Amin-Rasip, S., Dumitriu, S., Klootwijk, E., Knoers, N., Bockenbauer, D., Kleta, R., & Zdebik, A. A. (2013). KCNJ10 mutations display differential sensitivity to heteromerisation with KCNJ16. *Nephron Physiology*, 123(3-4), 7-14. <https://doi.org/10.1159/000356353>

### **Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### **Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

# KCNJ10 Mutations Display Differential Sensitivity to Heteromerisation with KCNJ16

Sophie Parrock<sup>a</sup> Sofia Hussain<sup>a, c, e</sup> Naomi Issler<sup>b, c</sup> Ann-Marie Differ<sup>d</sup>  
Nicholas Lench<sup>d</sup> Stefano Guarino<sup>f</sup> Michiel J.S. Oosterveld<sup>g</sup> Mandy Keijzer-Veen<sup>h</sup>  
Eva Brilstra<sup>h</sup> Hester van Wieringen<sup>i</sup> A. Yvette Konijnenberg<sup>h</sup> Sarah Amin-Rasip<sup>a</sup>  
Simona Dumitriu<sup>c, j</sup> Enriko Klootwijk<sup>c</sup> Nine Knoers<sup>h</sup> Detlef Bockenhauer<sup>b–d</sup>  
Robert Kleta<sup>a–d</sup> Anselm A. Zdebik<sup>a, c</sup>

<sup>a</sup>Department of Neuroscience, Physiology and Pharmacology and <sup>b</sup>Institute of Child Health, University College London, <sup>c</sup>Centre for Nephrology, University College London, Royal Free Hospital, and <sup>d</sup>Great Ormond Street Hospital for Children, London, UK; <sup>e</sup>Quaid-i-Azam University, Islamabad, Pakistan; <sup>f</sup>Department of Pediatrics, Second University of Naples, Naples, Italy; <sup>g</sup>Emma Children's Hospital Amsterdam, Amsterdam, <sup>h</sup>Department of Medical Genetics, Division Biomedical Genetics, University Medical Centre Utrecht, Utrecht, and <sup>i</sup>Pediatrics, St. Antonius Hospital, Nieuwegein, The Netherlands; <sup>j</sup>University of Medicine and Pharmacy, Victor Babes, Timisoara, Romania

## Key Words

EAST syndrome · Gitelman syndrome · SeSAME · KCNJ10 · Kir4.1 · Tubulopathy · Inward rectifier · Potassium channel · Kidney

## Abstract

**Background/Aims:** Mutations in the inwardly-rectifying K<sup>+</sup>-channel KCNJ10/Kir4.1 cause autosomal recessive EAST syndrome (epilepsy, ataxia, sensorineural deafness and tubulopathy). KCNJ10 is expressed in the distal convoluted tubule of the kidney, stria vascularis of the inner ear and brain glial cells. Patients diagnosed clinically with EAST syndrome were genotyped and mutations in KCNJ10 were studied functionally. **Methods:** Patient DNA was amplified and sequenced, and new mutations were identified. Mutant and wild-type KCNJ10 constructs were cloned and heterologously expressed in *Xenopus* oocytes. Whole-cell K<sup>+</sup> currents were measured by 2-electrode voltage clamping and chan-

nel expression was analysed by Western blotting. **Results:** We identified 3 homozygous mutations in *KCNJ10* (p.F75C, p.A167V and p.V91fs197X), with mutation p.A167V previously reported in a compound heterozygous state. Oocytes expressing wild-type human KCNJ10 showed inwardly rectified currents, which were significantly reduced in all of the mutants ( $p < 0.001$ ). Specific inhibition of KCNJ10 currents by Ba<sup>2+</sup> demonstrated a large residual function in p.A167V only, which was not compatible with causing disease. However, co-expression with KCNJ16 abolished function in these heteromeric channels almost completely. **Conclusion:** This study provides an explanation for the pathophysiology of the p.A167V KCNJ10 mutation, which had previously not been considered pathogenic on its own. These findings provide evidence for the functional cooperation of KCNJ10 and KCNJ16. Thus, in vitro ascertainment of KCNJ10 function may necessitate co-expression with KCNJ16.

© 2013 S. Karger AG, Basel

## Introduction

Defective renal tubular ion transport leads to distinct salt-losing nephropathies, and the study of these diseases has revealed important insights into renal physiology [1]. EAST (epilepsy, ataxia, sensorineural deafness and renal tubulopathy) syndrome comprises a salt-losing tubulopathy similar to Gitelman syndrome with salt-wasting, activation of the renin-angiotensin-aldosterone system, hypokalaemic metabolic alkalosis, hypomagnesaemia and hypocalciuria, and is caused by loss of function of KCNJ10/Kir4.1 [2, 3].

Here, we present 3 (including 2 new) mutations identified in 4 patients from 3 consanguineous families and describe the consequences for KCNJ10 function in the oocyte expression system. Our data provide new insights into mutant KCNJ10 assembly and function, as one mutation, p.A167V, had been previously described as non-pathogenic on its own due to its mild effect on KCNJ10-mediated currents [4].

KCNJ10 functions either as a homotetramer or forms heterotetramers with the related channel KCNJ16 [5]. In the renal tubule, KCNJ10 localizes to the basolateral membrane of distal convoluted tubular epithelial cells [6], and supplies  $K^+$  to the basolateral  $Na^+/K^+$ -ATPase that powers transcellular electrolyte transport. Hans Ussing initially described this mechanism as pump-leak coupling [7]. In the distal convoluted tubule, KCNJ10 also maintains the driving force for basolateral  $Cl^-$  exit by hyperpolarizing the basolateral membrane, and thus favours transcellular reabsorption of  $Mg^{2+}$  [8]. Consequently, patients with mutations in this gene, and *KCNJ10*<sup>-/-</sup> mice, also show defective handling of electrolytes other than sodium [2].

In the ear, KCNJ10 is expressed in the stria vascularis which secretes  $K^+$  into the endolymph [9] and is required to generate the 'endocochlear potential' of around +100 mV, largely attributable to a  $K^+$  diffusion potential across the apical membrane of stria intermediate cells, which is essential for hearing [10].

In the brain and eyes, KCNJ10 is expressed in astroglia and retinal Müller cells, and helps maintain the ionic environment of the extracellular space by providing 'spatial buffering' of  $K^+$  by siphoning  $K^+$  from regions of high  $[K^+]_o$ , generated by neuronal repolarisation, to places of low  $[K^+]_o$  such as neighbouring blood vessels [11]. Inwardly rectifying  $K^+$  channels may facilitate the uptake of  $K^+$  into depolarized glial cells [12, 13] as well as its release at sites of lower extracellular potassium [14] because glial cells are electrically coupled.

*KCNJ10*<sup>-/-</sup> mice show hearing loss, ataxia and premature death, the latter a consequence of hypomyelination and degeneration of the spinal cord [15], as well as changes in  $Na^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  handling. *KCNJ10*<sup>-/-</sup> mice also show a loss of the slow PIII response of the light-evoked electroretinogram, which is generated by  $K^+$  fluxes in Müller cells expressing KCNJ10 and KCNJ16 [16]. We recently demonstrated similar findings in patients with EAST syndrome [17]. When KCNJ10 is specifically disrupted in astroglia, mice show stress-induced seizures and severe ataxia [18]. Glial cells are strongly depolarized, supporting the role of KCNJ10 in clearing  $K^+$  from the extracellular space, which has recently been shown directly using  $K^+$ -selective micro-electrodes [19].

Mutations previously discovered in the coding region of *KCNJ10* include the homozygous missense mutations p.R65P, p.G77R, p.C140R and p.T164I [2, 3]. Compound heterozygous missense/nonsense mutations p.R65P and p.R199X and compound heterozygous missense mutations p.A167V and p.R297C were found in two other kindreds [3]. Table 1 compiles all functionally characterized mutations in *KCNJ10* published so far.

The comparative effects of some *KCNJ10* mutations were analysed at the whole-cell level and replicated at the single-channel level, with strongly reduced open probability in R65P and R175Q and almost absent channel activity in G77R [20]. Excised patches showed a dramatic shift of the pH sensitivity in the mutant channels R65P and R175Q, with some residual channel activity at unphysiologically high pH. A study of heterologously expressed mutant channels found less residual conductance when intracellular pH was reduced from 7.4 to 6.8, which showed a minimal effect on wild-type (WT) KCNJ10 [4]. Due to undetectable currents, the truncated KCNJ10 R199X could not be investigated in isolation [20]. We recently investigated 3 partially functional mutations: p.R65C (c.193C>T) identified in 1 patient, a homozygous missense mutation p.F75L (c.225T>G) identified in 2 affected siblings and a homozygous missense mutation p.R297C (c.889C>T) identified in 1 patient [21]. A frame-shift mutation p.V259fs259X resulted in complete loss of function [21].

We now describe further mutations found in patients clinically diagnosed with EAST syndrome. The mutations were cloned and heterologously expressed in *Xenopus laevis* oocytes to investigate the function of mutant KCNJ10 by 2-electrode voltage clamping.

**Table 1.** A compilation of KCNJ10 mutations on which functional data have been published to date

Mutation	Electrophysiological effect in oocytes	Electrophysiological effect in mammalian cells	Surface expression	Refs.	Origin
p.T57I	loss of function		at surface	[35]	Somalian
p.R65C	~20% function			[21]	Algerian
p.R65P	~20% function	partially functional	at surface	[2, 4, 20]	Pakistani
p.F75L	<10% function			[21]	Afro-Caribbean
p.G77R	<5% function	partially functional	at surface, but reduced	[2, 4, 20]	UAE
p.C140R		loss of function	at surface, but reduced	[4, 30]	?
p.T164I		loss of function	at surface	[4, 30]	?
p.A167V	~65% function	~65% function	at surface, but reduced	[4, 30]	Italian/Dutch (Turkish origin)
p.R175Q	<5% function		at surface	[20]	Iranian
p.R199X		loss of function	loss of surface expression but increased surface expression	[30]	?
				[4]	
p.V259X	loss of function			[21]	Indian
p.R297C	<10% function	~5% function	at surface	[4, 21, 30]	Iranian

## Materials and Methods

### Genomic DNA Sequencing

Genetic studies were approved by the Institute of Child Health-Great Ormond Street Hospital Research Ethics Committee, after obtaining written consent. The entire single coding exon and adjacent intronic sequence of *KCNJ10* was sequenced by the Sanger method using a Beckman Coulter CEQ8000 or an ABI prism 377 sequencer. PCR primer sequences can be supplied on request. Ensembl gene transcript ENST00000368089 was used as a reference.

### Cloning of Mutant *KCNJ10*

Mutant *KCNJ10* was inserted into the pTLB oocyte expression vector after PCR amplification of the single coding exon with primers introducing Xho I and Xba I restriction sites, maintaining the Kozak sequence [22], using Phusion polymerase (NEB, UK). Ligated plasmids were transformed into *Escherichia coli* XL1 blue (Stratagene, USA), and plasmid DNA was purified using NucleoSpin® columns (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations. cDNA inserts of all plasmids were fully sequence verified.

### Heterologous Expression of *KCNJ10*

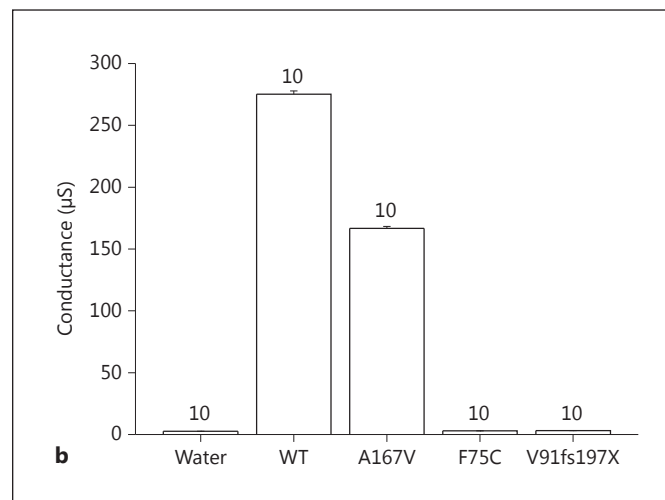
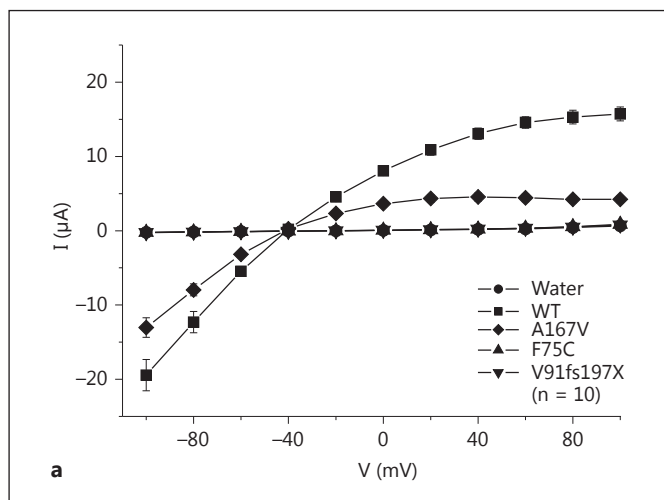
Plasmids containing mutant and WT *KCNJ10* [2] were linearized by Mlu I digestion (Fermentas, UK), and cRNA was synthesized using the mMESSAGE mMACHINE® SP6 Kit (Ambion, UK). Adult female *Xenopus laevis* (University of Portsmouth) were sacrificed under Home Office-licensed Schedule 1 of the Animals (Scientific Procedures) Act 1986, and the ovaries were dissected and digested with collagenase (Type II, Invitrogen) dissolved at 1–2 mg/ml in ND96, containing (in mM): 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub> · 2H<sub>2</sub>O, 1 MgCl<sub>2</sub> · 6H<sub>2</sub>O, 5 HEPES and pH 7.5 (NaOH). Healthy-looking stage V and VI oocytes [23] were selected for injection with 1–2 ng WT or mutant *KCNJ10* cRNA and then incubated for 1–2 days at 17°C.

### Western Blotting

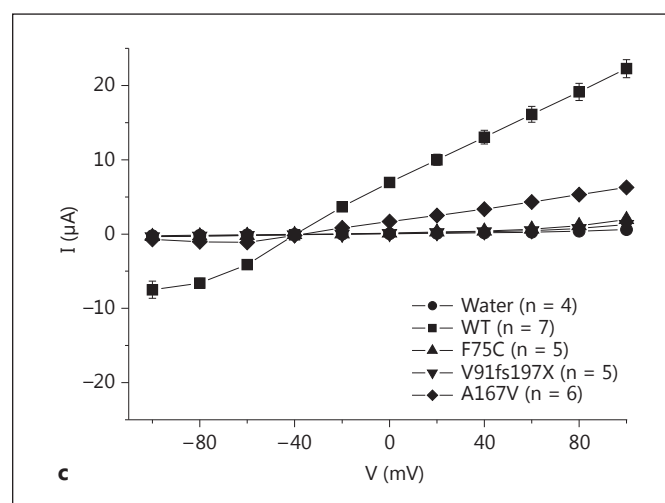
Membrane fractions were extracted by trituration and 2 centrifugation steps at 4,000 g for 3 min to remove yolk and lipids from 10 oocytes each, and then injected with mutant, WT or control. The intermediate phase was denatured in SDS-containing loading buffer and run on 8.5 or 10% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Trans-Blot Turbo pack, BioRad, UK) using a semidry blotter (BioRad, UK). Membranes were stained with 0.2% Ponceau's stain in 5% acetic acid for 5 min, washed and imaged. The membrane was blocked and incubated with the *KCNJ10* monoclonal antibody 1C11 (Sigma, UK) or a polyclonal antibody against *KCNJ16* [20]. Bound primary antibodies were detected by secondary anti-mouse or anti-rabbit antibodies coupled to peroxidase and enhanced chemiluminescence using home-made reagents and imaged using a Dyversity gel analyser and Genesnap software (Syngene, UK).

### Voltage Clamping

Voltage clamping was performed with a TEC01C amplifier (NPI Electronic GmbH, Tamm, Germany). Oocytes were perfused with ND96 with 18 mM KCl added (in order to increase specific currents as Kir channel conductance increases with [E<sub>K+</sub>]<sub>o</sub> [22, 23]) in an RC-1Z Oocyte Chamber (Harvard Apparatus, UK). Micro-electrodes were pulled from 1.5-mm glass capillaries with filament (Harvard Apparatus) using a DMZ-universal puller (Zeitz, Augsburg, Germany), and had a resistance of <1 MΩ when filled with 3 M KCl. Recordings were controlled using Strathclyde WinWCP and a PCI-6251 interface (National Instruments, UK), programmed at clamp steps from +100 to –100 mV in 20 mV decrements, each lasting 500 ms. The baseline holding voltage was held at –40 mV, close to E<sub>K+</sub>. 100 μM BaCl<sub>2</sub> was used as a reversible specific inhibitor of inwardly rectifying K<sup>+</sup> channels [24]. Recordings were analysed and plotted using Strathclyde electrophysiology software (written and supported by John Dempster, Strathclyde University, UK) and Origin (OriginLab, Northampton, Mass., USA).



**Fig. 1. a** Averaged current/voltage curves obtained in *Xenopus* oocytes injected with 2 ng WT or mutant KCNJ10 cRNA. Oocytes expressing KCNJ10 F75C and V91fs197X are indistinguishable from water-injected oocytes. Oocytes were held in 20 mM K<sup>+</sup> at -40 mV and clamped from +100 to -100 mV in 20 mV steps. Note that currents reversed around -40 mV due to the 20-mM K<sup>+</sup> solution used. Three batches of oocytes were analysed. **b** Analysis of the chord conductance between -100 and 0 mV. **c** Barium (100 μM) inhibited more strongly the inward component of WT and A167V mutant currents.



#### Statistical Analysis

Statistical analysis was performed using Origin. One-sided and two-sided Student's *t* tests were used as appropriate for paired and unpaired data, and *p* < 0.05 was considered significant. All data are given as means ± SEM.

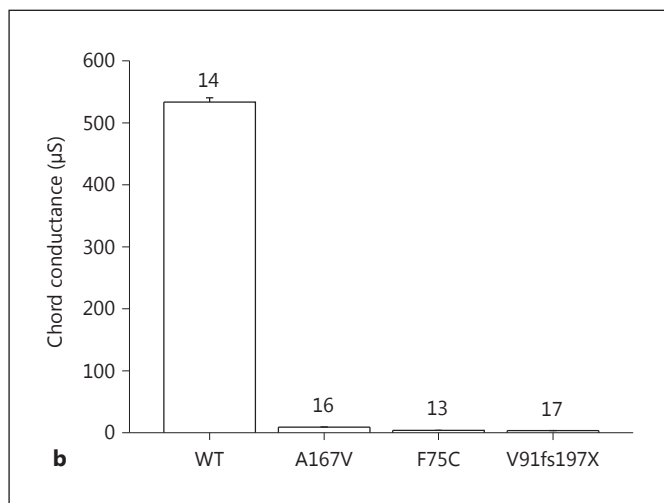
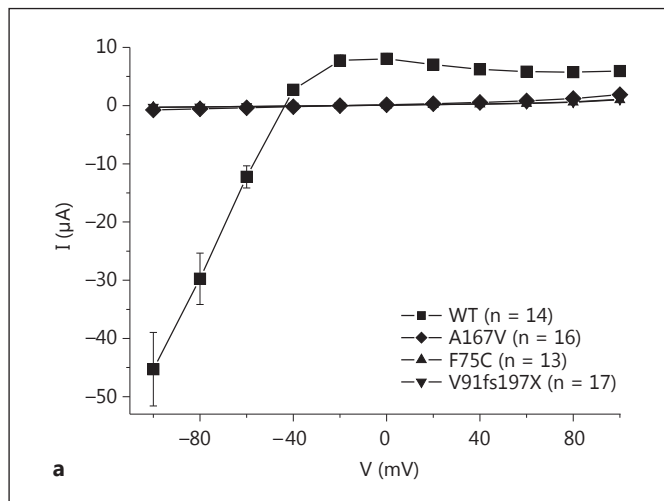
## Results

We identified 2 new mutations, p.F75C and p.V91fs197X (p.V91Gfs197X, due to a c.272delT mutation at cRNA level), and a known mutation p.A167V [4] in 4 consanguineous families. As expected, individuals affected with EAST syndrome showed homozygous mutations. Interestingly, the p.A167V, previously reported to be disease-causing only when paired with another more

severe mutation, was also found to be homozygous, and no other mutations were detected in the entire coding sequence and adjacent splice sites. We therefore studied this mutation in more detail.

As a first screen, we expressed all mutant channels along with water and WT controls in *Xenopus* oocytes. Currents in oocytes expressing KCNJ10 F75C and V91fs197X were indistinguishable from endogenous currents observed in water-injected oocytes (fig. 1a). As described previously [4], KCNJ10 A167V led to a mild reduction in current as compared to WT (to approx. 60%). This is also clearly seen in the analysis of chord conductances (-100; 0 mV) depicted in figure 1b. In a separate batch of oocytes, we analysed the barium block of these conductances (fig. 1c). Although there is a trend for some current remaining with submaximal barium (100 μM,

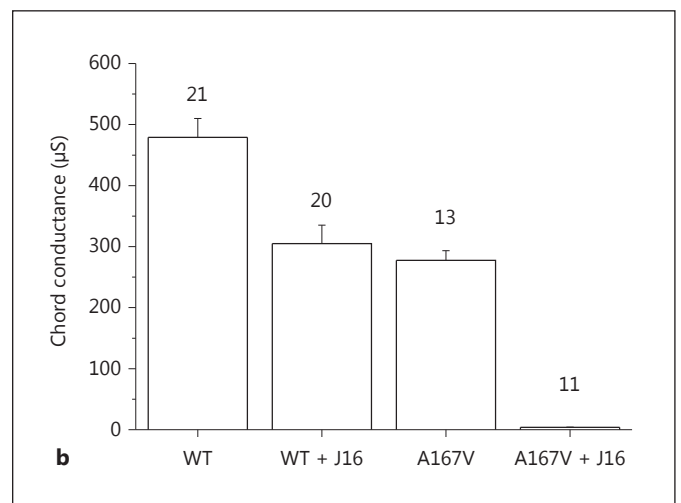
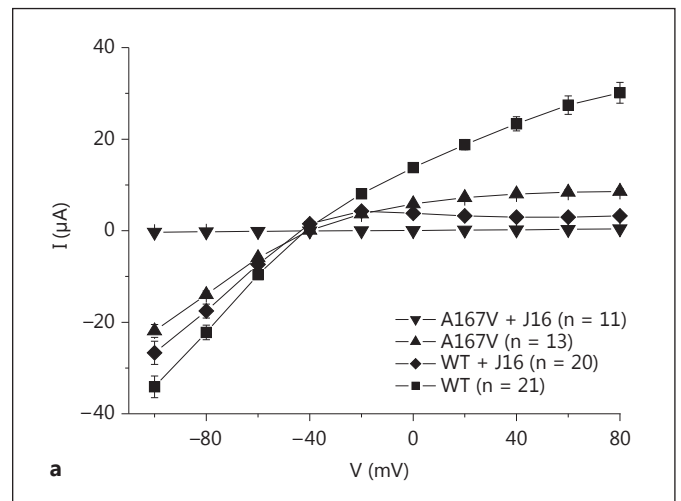




**Fig. 2. a** Averaged current/voltage curves obtained in *Xenopus* oocytes injected with 2 ng WT or mutant KCNJ10 cRNA and 4 ng KCNJ16 cRNA. Note the more pronounced inward rectification in WT KCNJ10/16 heteromeric channels ('WT' in the figure). KCNJ10 A167V/KCNJ16 ('A167V') showed current significantly different from the 2 non-functional mutations ('F75C' and 'V91fs197X'), but these currents were so low that currents from all mutations virtually superimpose. **b** Analysis of the chord conductance between -100 and 0 mV. Conductances as a percentage of WT KCNJ10: A167V, 1.7%; F75C, 0.7%; V91fs197X, 0.7%. The latter 2 are consistent with conductances endogenous to the oocyte.

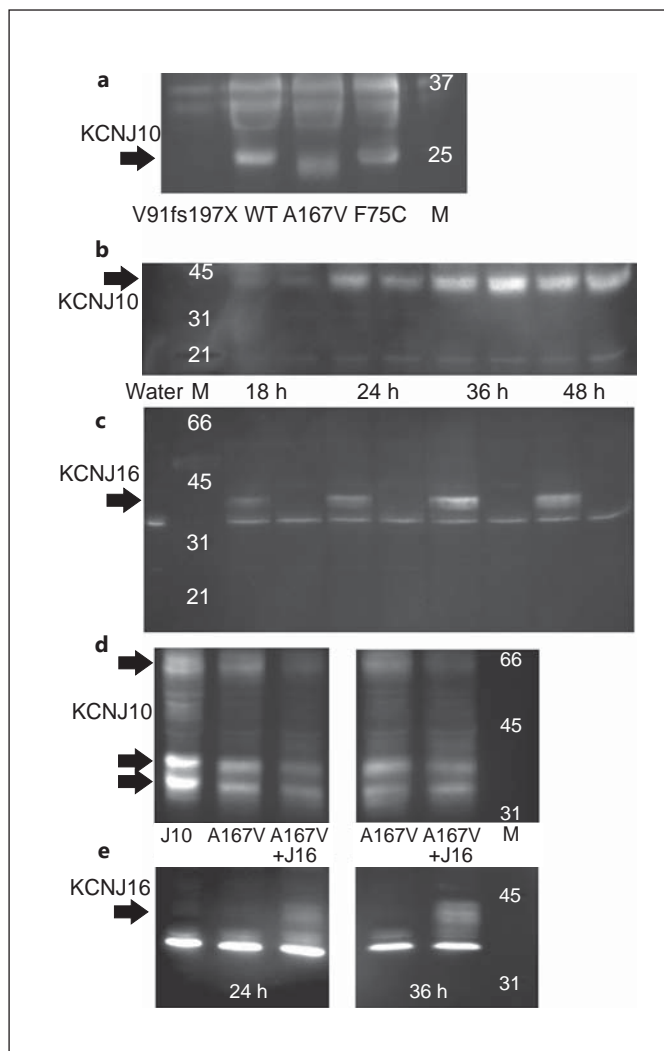
with 20 mM K<sup>+</sup>) in KCNJ10 F75C and V91fs197X, only WT and A167V-expressing oocytes showed significant block in the negative voltage range, as expected for a pore blocker.

EAST syndrome in a patient with a homozygous mutation exhibiting 60% residual function poses a genetic di-



**Fig. 3. a** Averaged current/voltage curves obtained in *Xenopus* oocytes expressing WT and KCNJ10 A167V (1 ng) with and without KCNJ16 (3 ng) in the same batch of oocytes. Again, altered rectification for WT and almost complete loss of current for KCNJ10 A167V upon co-expression with KCNJ16 was observed. **b** Analysis of the chord conductance between -100 and 0 mV. Conductances as a percentage of WT KCNJ10 alone: KCNJ10/KCNJ16, 64%; KCNJ10 A167V alone, 58%; KCNJ10 A167V/KCNJ16, 0.8%.

lemma (parents heterozygous for p.F75C or p.V91fs197X would be expected to have an even greater degree of functional impairment, yet are asymptomatic). KCNJ10 either forms homotetramers or heterotetramers with the related channel KCNJ16 [5]. We therefore studied heteromeric KCNJ10/KCNJ16 channels. Co-expression of an excess



**Fig. 4.** **a** KCNJ10 WT, A167V, F75C and V91fs197X expressed in oocytes, separated by SDS-PAGE and probed for KCNJ10. Note that V91fs197X cannot be detected with our antibody as it recognizes an epitope lost through frameshift in this mutation, suggesting the antibody is specific. **b, c** Time-course of expression of 1 ng KCNJ10 and 3 ng KCNJ16 cRNA co-expressed in oocytes over 48 h. Detection in membrane preparations with a monoclonal antibody against KCNJ10 (**b**), and with a polyclonal antibody against KCNJ16 (**c**). **d** Western blot probed for KCNJ10 prepared from oocytes expressing KCNJ10 A167V with and without KCNJ16. **e** Same membrane as in **d**, probed for KCNJ16. Expression levels appear somewhat lower for KCNJ10 A167V, but the co-expression with KCNJ16 does not alter expression significantly, suggesting that overall stability of KCNJ10 A167V is unaltered by heteromerisation with KCNJ16. KCNJ10 is known to produce multiple bands on Western blotting, depending on the preparation [34]. Note that the lane description applies for **d** and **e**. Arrows indicate specific bands throughout the figure, and M indicates molecular weight marker lanes with the approximate molecular weight given in kDa.

of KCNJ16 cRNA resulted in currents with much stronger inward rectification for WT and failed to rescue F75C and V91fs197X mutant KCNJ10. Interestingly, currents were almost completely abolished in oocytes expressing KCNJ10 A167V/KCNJ16 heteromeric channels (fig. 2a). Less than 2% of the current remained, as is more clearly seen in the analysis of chord conductances (fig. 2b).

We next confirmed the effect of KCNJ16 co-expression on KCNJ10 A167V in the same batch of oocytes. These results are summarized in figure 3. Whereas co-expression of KCNJ16 with WT KCNJ10 (injected at 3:1 cRNA stoichiometry) merely altered rectification, it almost completely abolished currents in oocytes expressing KCNJ10 A167V/KCNJ16 heteromeric channels (fig. 3a). This is clearly reflected in the chord conductance analysis shown in figure 3b.

The next experiments addressed the stability of mutant KCNJ10. The expression level of KCNJ10 was similar in oocytes injected with WT and mutant KCNJ10 cRNA (fig. 4a), except for KCNJ10 V91fs197X in which the epitope is absent and the channel can therefore not be detected by our antibody. In order to assess the relative time course of expression for KCNJ10 and KCNJ16, we first analysed expression level at 18, 24, 36 and 48 h. Both channels appear to peak around 36 h (fig. 4a, KCNJ10; fig. 4b, KCNJ16). Note that the band in figure 4b running at approximately 35 kDa and present in all samples is non-specific (also present in water-injected oocytes) and indicates equal loading of all lanes. Co-expression of KCNJ16 (fig. 4e) with KCNJ10 A167V at two different time points did not affect overall stability of KCNJ10 A167V (fig. 4d, same blot as in e, but reprobed for KCNJ10). Note that the band present in all samples in figure 4e is non-specific and indicates equal loading.

## Discussion

Bartter's syndromes result from mutations in transporters and ion channels in the loop of Henle, which function in a concerted fashion. More distally, the generally milder Gitelman syndrome results from mutations in the NaCl cotransporter mainly expressed in the distal convoluted tubule [25], and pseudohypoaldosteronism can result from mutations in the epithelial sodium channel [26]. Loss of KCNJ10/Kir4.1 causes an autosomal recessive salt-losing renal tubulopathy similar to Gitelman syndrome with salt-wasting, activation of the renin-angiotensin-aldosterone system, hypokalaemic metabolic alkalosis, hypomagnesaemia and hypocalciuria [2, 3]. In

mice, there is also expression of KCNJ10 in the connecting tubule, and in the cortical collecting duct. In humans there may also be expression in the cortical thick ascending limb of the loop of Henle [27, 28]. In addition to these renal tubule segments, KCNJ10 is also expressed in the brain, inner ear and eyes, and mutations in this channel can lead to EAST syndrome [2].

Multiple pathogenic *KCNJ10* mutations have been previously described in patients with EAST syndrome. Some mutations have residual function (p.A167V>p.R65P>p.R175Q>p.G77R) [2, 21], as summarized in table 1. p.R199X, a nonsense mutation, displayed complete loss of function [4], but was only identified in a compound heterozygous patient.

We previously described mutations with severely impaired function (p.R297C seen for the first time in a homozygous state, and 3 new mutations: p.R65C, p.F75L, p.V259X) [21]. Three of these mutated channels had residual function (R65C>F75L>R297C) [21].

In the present study, we describe a p.F75C substitution, which, consistent with a less conservative exchange compared to p.F75L, led to a complete loss of function. The results were very similar for the early frameshift mutation p.V91fs197X. We cannot exclude residual function at 1–2% of WT levels, due to limitations of the expression system. However, it is questionable whether less than 2% residual function would be physiologically relevant.

We confirmed strong residual function in KCNJ10 A167V, a mutation which has been described previously [4, 29, 30]. Based on genetic evidence available at the time, it was hypothesized that this mutation could only cause disease when paired with a functionally more severe mutation [29, 30]. However, we now found this mutation in a homozygous state in 3 patients with EAST syndrome, although no epileptic seizures have been described in 2 patients from one family. Infrequent epileptic episodes can be overlooked, so at this point it is unclear whether these patients have genuine absence of epilepsy, or whether it has not been recognized. The patients do show hypokalaemic alkalosis, hypomagnesaemia, sensorineural hearing loss and ataxia, which are hallmarks of EAST syndrome in all 3 affected organ systems. As the parents of patients with loss-of-function mutations are asymptomatic, we were initially puzzled by this finding, as KCNJ10 A167V alone shows >60% of WT function. KCNJ10 has been reported to form heteromeric channels with KCNJ16 both in expression systems [5], native astrocytes [31] and the kidney distal convoluted tubule [27, 32]. KCNJ16 does not mediate currents on its own, but increases inward rectification in KCNJ10/KCNJ16 hetero-

meric channels [5]. We therefore hypothesized that KCNJ16 co-expression might reduce function of KCNJ10 A167V. Indeed, we found a very strong reduction of currents in KCNJ10 A167V/KCNJ16 heteromeric channels. This provides a rationale for how brain and kidney phenotypes could develop – in the absence of strong functional defects of homomeric KCNJ10 A167V. Although decreased surface expression has been described for this mutation [29, 30], this effect was much weaker than what we would expect from our functional data on heteromeric channels. As overall expression was unchanged, and not affected by KCNJ16 co-expression, we hypothesize that heteromeric channels are less likely to traffic to the surface.

Interestingly, although KCNJ16 is expressed in the inner ear, it has not been shown to be expressed in strial intermediate cells, where KCNJ10 resides in the apical membrane and generates the strong positive endocochlear potential essential for the hearing process [9, 10]. The endocochlear potential, together with the high K<sup>+</sup> concentration present in the endolymph, makes K<sup>+</sup> enter cochlear hair cells through mechanosensitive channels. KCNJ16 is rather expressed in cochlear wall fibrocytes [33]. We do not have a satisfactory explanation for the hearing loss observed in patients with a homozygous p.A167V mutation. However, we speculate that co-expression of other channels present in these cells may have a similar effect as co-expression of KCNJ16 described here.

In summary, we describe 2 new mutations in KCNJ10, both of which lead to loss of function, within the resolution of our technique. We also provide an explanation for the pathogenicity of KCNJ10 mutation p.A167V, which has previously been thought to be a disease modifier in the context of another, more severe mutation.

## Acknowledgements

A.A.Z. received support from the Peter Samuel Trust Fund and St. Peter's Trust for Kidney, Bladder and Prostate Research. D.B. and R.K. received support from the European Union, FP7 (grant agreement 2012-305608 'European Consortium for High-Throughput Research in Rare Kidney Diseases (EURenOmics)'). S.P. and S.A.-R. are undergraduate students at UCL and contributed to the study as part of their (i)BSc research projects supervised by A.A.Z. S.H. was a visiting PhD student from Quaid-i-Azam University, Islamabad, Pakistan. We thank Dr. Maryam Khosravi for critically reading the manuscript.



## References

- Kleta R, Bockenhauer D: Bartter syndromes and other salt-losing tubulopathies. *Nephron Physiol* 2006;104:p73–p80.
- Bockenhauer D, Feather S, Stanescu HC, Bandulik S, Zdebek AA, Reichold M, Tobin J, Lieberer E, Sterner C, Landoure G, Arora R, Sirimanna T, Thompson D, Cross JH, van't Hoff W, Al Masri O, Tullus K, Yeung S, Anikster Y, Klootwijk E, Hubank M, Dillon MJ, Heitzmann D, Arcos-Burgos M, Knepper MA, Dobbie A, Gahl WA, Warth R, Sheridan E, Kleta R: Epilepsy, ataxia, sensorineural deafness, tubulopathy, and KCNJ10 mutations. *N Engl J Med* 2009;360:1960–1970.
- Scholl UI, Choi M, Liu T, Ramaekers VT, Hausler MG, Grimmer J, Tobe SW, Farhi A, Nelson-Williams C, Lifton RP: Seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by mutations in KCNJ10. *Proc Natl Acad Sci USA* 2009;106:5842–5847.
- Williams DM, Lopes CM, Rosenhouse-Dantsker A, Connelly HL, Matavel A, O-Uchi J, McBeath E, Gray DA: Molecular basis of decreased Kir4.1 function in SeSAME/EAST syndrome. *J Am Soc Nephrol* 2010;21:2117–2129.
- Pessia M, Tucker SJ, Lee K, Bond CT, Adelman JP: Subunit positional effects revealed by novel heteromeric inwardly rectifying K<sup>+</sup> channels. *EMBO J* 1996;15:2980–2987.
- Ito M, Inanobe A, Horio Y, Hibino H, Iso moto S, Ito H, Mori K, Tonosaki A, Tomoike H, Kurachi Y: Immunolocalization of an inwardly rectifying K<sup>+</sup> channel, K(AB)-2 (Kir4.1), in the basolateral membrane of renal distal tubular epithelia. *FEBS Lett* 1996;388:11–15.
- Schultz SG: Pump-leak parallelism in sodium-absorbing epithelia: the role of ATP-regulated potassium channels. *J Exp Zool* 1997;279:476–483.
- Xi Q, Hoenderop JG, Bindels RJ: Regulation of magnesium reabsorption in DCT. *Pflügers Arch* 2009;458:89–98.
- Marcus DC, Wu T, Wangemann P, Kofuji P: KCNJ10 (Kir4.1) potassium channel knock-out abolishes endocochlear potential. *Am J Physiol Cell Physiol* 2002;282:C403–C407.
- Zdebek AA, Wangemann P, Jentsch TJ: Potassium ion movement in the inner ear: insights from genetic disease and mouse models. *Physiology (Bethesda)* 2009;24:307–316.
- Higashi K, Fujita A, Inanobe A, Tanemoto M, Doi K, Kubo T, Kurachi Y: An inwardly rectifying K(+) channel, Kir4.1, expressed in astrocytes surrounds synapses and blood vessels in brain. *Am J Physiol Cell Physiol* 2001;281:C922–C931.
- Butt AM, Kalsi A: Inwardly rectifying potassium channels (Kir) in central nervous system glia: a special role for Kir4.1 in glial functions. *J Cell Mol Med* 2006;10:33–44.
- Olsen ML, Sontheimer H: Functional implications for Kir4.1 channels in glial biology: from K<sup>+</sup> buffering to cell differentiation. *J Neurochem* 2008;107:589–601.
- Orkand RK: Glial-interstitial fluid exchange. *Ann NY Acad Sci* 1986;481:269–272.
- Neusch C, Rozengurt N, Jacobs RE, Lester HA, Kofuji P: Kir4.1 potassium channel subunit is crucial for oligodendrocyte development and in vivo myelination. *J Neurosci* 2001;21:5429–5438.
- Kofuji P, Ceelen P, Zahs KR, Surbeck LW, Lester HA, Newman EA: Genetic inactivation of an inwardly rectifying potassium channel (Kir4.1 subunit) in mice: phenotypic impact in retina. *J Neurosci* 2000;20:5733–5740.
- Thompson DA, Feather S, Stanescu HC, Freudenthal B, Zdebek AA, Warth R, Ognjanovic M, Hulton SA, Wassmer E, Van't Hoff W, Russell-Eggitt I, Dobbie A, Sheridan E, Kleta R, Bockenhauer D: Altered electroretinograms in patients with KCNJ10 mutations and east syndrome. *J Physiol* 2011;589:1681–1689.
- Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD: Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. *J Neurosci* 2007;27:11354–11365.
- Haj-Yasein NN, Jensen V, Vindedal GF, Gundersen GA, Klungland A, Ottersen OP, Hvalby O, Nagelhus EA: Evidence that compromised K<sup>+</sup> spatial buffering contributes to the epileptogenic effect of mutations in the human Kir4.1 gene (KCNJ10). *Glia* 2011;59:1635–1642.
- Reichold M, Zdebek AA, Lieberer E, Rapedius M, Schmidt K, Bandulik S, Sterner C, Tegtmeyer I, Penton D, Baukrowitz T, Hulton SA, Witzgall R, Ben-Zeev B, Howie AJ, Kleta R, Bockenhauer D, Warth R: KCNJ10 gene mutations causing EAST syndrome (epilepsy, ataxia, sensorineural deafness, and tubulopathy) disrupt channel function. *Proc Natl Acad Sci USA* 2010;107:14490–14495.
- Freudenthal B, Kulaveerasingam D, Lingappa L, Shah MA, Brueton L, Wassmer E, Ognjanovic M, Dorison N, Reichold M, Bockenhauer D, Kleta R, Zdebek AA: KCNJ10 mutations disrupt function in patients with EAST syndrome. *Nephron Physiol* 2011;119:p40–p48.
- Lopatin AN, Nichols CG: [K<sup>+</sup>] dependence of open-channel conductance in cloned inward rectifier potassium channels (IRK1, Kir2.1). *Biophys J* 1996;71:682–694.
- Hagiwara S, Miyazaki S, Rosenthal NP: Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *J Gen Physiol* 1976;67:621–638.
- Hagiwara S, Miyazaki S, Moody W, Patlak J: Blocking effects of barium and hydrogen ions on the potassium current during anomalous rectification in the starfish egg. *J Physiol* 1978;279:167–185.
- Simon DB, Nelson-Williams C, Bia MJ, Ellison D, Karet FE, Molina AM, Vaara I, Iwata F, Cushner HM, Koolen M, Gainza FJ, Gitelman HJ, Lifton RP: Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 1996;12:24–30.
- Chang SS, Grunder S, Hanukoglu A, Rosler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC, Lifton RP: Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1. *Nat Genet* 1996;12:248–253.
- Lachheb S, Cluzeaud F, Bens M, Genete M, Hibino H, Lourdel S, Kurachi Y, Vandewalle A, Teulon J, Paulais M: Kir4.1/Kir5.1 channel forms the major K<sup>+</sup> channel in the basolateral membrane of mouse renal collecting duct principal cells. *Am J Physiol* 2008;294:F1398–F1407.
- Lourdel S, Paulais M, Cluzeaud F, Bens M, Tanemoto M, Kurachi Y, Vandewalle A, Teulon J: An inward rectifier K(+) channel at the basolateral membrane of the mouse distal convoluted tubule: similarities with Kir4-Kir5.1 heteromeric channels. *J Physiol* 2002;538:391–404.
- Sala-Rabanal M, Kucheryavykh LY, Skatchkov SN, Eaton MJ, Nichols CG: Molecular mechanisms of EAST/SeSAME syndrome mutations in Kir4.1 (KCNJ10). *J Biol Chem* 2010;285:36040–36048.
- Tang X, Hang D, Sand A, Kofuji P: Variable loss of Kir4.1 channel function in sesame syndrome mutations. *Biochem Biophys Res Commun* 2010;399:537–541.
- Hibino H, Fujita A, Iwai K, Yamada M, Kurachi Y: Differential assembly of inwardly rectifying K<sup>+</sup> channel subunits, Kir4.1 and Kir5.1, in brain astrocytes. *J Biol Chem* 2004;279:44065–44073.
- Paulais M, Bloch-Faure M, Picard N, Jacques T, Ramakrishnan SK, Keck M, Sohet F, Eladari D, Houillier P, Lourdel S, Teulon J, Tucker SJ: Renal phenotype in mice lacking the Kir5.1 (KCNJ16) K<sup>+</sup> channel subunit contrasts with that observed in SeSAME/EAST syndrome. *Proc Natl Acad Sci USA* 2011;108:10361–10366.
- Hibino H, Higashi-Shingai K, Fujita A, Iwai K, Ishii M, Kurachi Y: Expression of an inwardly rectifying K<sup>+</sup> channel, Kir5.1, in specific types of fibrocytes in the cochlear lateral wall suggests its functional importance in the establishment of endocochlear potential. *Eur J Neurosci* 2004;19:76–84.
- Singh R, Wangemann P: Free radical stress-mediated loss of KCNJ10 protein expression in stria vascularis contributes to deafness in Pendred syndrome mouse model. *Am J Physiol* 2008;294:F139–F148.
- Scholl UI, Dave HB, Lu M, Farhi A, Nelson-Williams C, Listman JA, Lifton RP: SeSAME/East syndrome – phenotypic variability and delayed activity of the distal convoluted tubule. *Pediatr Nephrol* 2012;27:2081–2090.